Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity

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Abstract. A forerunning study on the relationship between antibodies to the protective antigen (PA) and lethal factor (LF) components of anthrax toxin and protective immunity has been expanded and extended to include the third toxin component, the edema factor (EF). It was found that protection against the "vaccine resistant" Ames strain was possible in the absence of detectable anti-LF and anti-EF antibodies. Evidence is given that PA may be the essential anthrax-derived antigen for protection, but that equally essential is that it be presented to the host's immune system in such a manner as to provide stimulation of more than just production of antibody to PA. Titers to the three components in sera of individuals with histories of clinically diagnosed anthrax as well as from human vaccinees are included in the report.

Introduction

The application of a competitive inhibition enzyme-linked immunosorbent assay (ELISA) to the detection of serum antibodies to Protective Antigen (PA) and Lethal Factor (LF) components of anthrax toxin has been described [7]. In conjunction with protection test studies in guinea pigs, it was noted that protection appeared to be a function of more than just antibody titers to PA and LF.

At that time, purified Edema Factor (EF), the third of the synergistic anthrax toxin components and a calmodulin dependent adenylate cyclase [3], was not available and its role in protective immunity could not be assessed.

Purification of EF free of LF and PA was subsequently achieved and examination of the relationship between anti-EF antibodies and protective immunity has now been carried out.

Materials and methods

Antigens

PA, LF, and EF were produced and purified as previously described [3, 4].

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Human vaccine sera

The sera examined were from 18 of the individuals included in the earlier study concerned only with PA and LF [7]. Twelve of these had received full courses of the UK vaccine (product licence 1511/0037, prepared for the UK Department of Health and Social Security in the Division of Biologics, PHLS Centre for Applied Microbiology and Research). The full course comprises an initial short course of three intramuscular 0.5 ml injections at 3-week intervals followed by a 6-month booster and, except in three instances, one or more annual boosters. The other 6 vaccinees had received only the short course of the US vaccine (US license no. 99, prepared by the Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich, USA).

Human case sera

Thirteen of the 77 sera originating from clinically diagnosed cases of anthrax in the Matabeleland district of Zimbabwe and included in the previous study [7] were re-examined. Nine of these were the samples still available which had been positive for anti-PA antibodies including three taken from one patient when first seen and then again 15 and 42 days later. The remaining four sera were randomly selected from those that had been anti-PA and anti-LF negative.

Guinea pigs sera

The guinea pigs from which sera included in this study were taken can be broadly divided into two groups.

Group I consisted of those included in the earlier study [7] and could be subdivided into six subgroups according to the vaccinations or challenges that they had received. Sera from six guinea pigs in each of the subgroups were re-examined in this study. Subgroups 1 and 2 had been vaccinated subcutaneously with 0.5 ml of the UK and US vaccines respectively on days 0, 14 and 28. Subgroup 3 had received a single dose (0.2 ml containing $\pm$ 5 x 10^6 spores) of live spore Sterne strain animal vaccine (Wellcome Laboratories, Beckenham, Kent, UK) and subgroup 4 had similarly received a single dose (0.2 ml containing $\pm$ 5 x 10^5 spores) of live spore vaccine derived from Russian STI strain (Tobol'sk Bioplant, Ministry of Agriculture, Moscow, USSR). Appropriate unvaccinated controls were monitored simultaneously.

The sera from animals in these groups were obtained prior to challenge on day 42. Subgroups 5 and 6 were subgroup 3 and 4 guinea pigs that survived initial challenge and these sera were collected just before re-challenge 6 weeks after the first challenge.

Group II guinea pigs represented a second series of vaccine protection trials. Apart from the appropriate unvaccinated controls, these animals had received the UK or the US vaccines with or without other additives according to the following schedules. On days 0, 14 and 28, subgroup A guinea pigs received 0.15 ml volumes of vaccine (UK or US) in the rear left flank

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and, in the rear right flank, 0.15 ml volumes of buffered saline or suspensions of either live vegetative *Bacillus cereus* strain F4810/62 (NCTC 11,143; 9 x 10^7 to 3 x 10^8 cfu/ml) or live vegetative *B. cereus* strain F4433/73 (NCTC 11,145; 4 x 10^7 to 2 x 10^8 cfu/ml). These strains were chosen on the basis of their well-characterised behaviour in various types of animal studies [6].

Subgroup B guinea pigs had similarly been vaccinated on days 0, 14 and 28 with UK or US vaccines (0.3 ml) supplemented with one of the following: (i) 0.3 ml phosphate buffered saline; (ii) 0.3 ml heat-killed [boiling water bath for 30 minutes] suspension of 2.5 x 10^9 cfu/ml *Corynebacterium ovis* strain OV 137C2; (iii) 0.3 ml Freund's complete adjuvant [Sigma Chemical Co., St. Louis, MO, USA;] (iv) soluble lyophilised saponin adjuvant Quil-A [Superfos Biosector, DK-2950, Vedbaek, Denmark], 66 μg/dose or (v) synthetic amorphous SiO_2 adjuvant Gasil-23D [Crosfield Chemicals, Warrington, Cheshire, UK], 7.5 mg/dose.

The sera were again collected prior to challenge 2 weeks after the third dose.

ELISA procedure

The same inhibition ELISA as described in the previous study [7] was used in this study. In the case of PA and LF, ELISA plates were again sensitized with solutions (60 μl/well) containing an estimated 15 μg/ml per millilitre of coating buffer (0.16 g Na_2CO_3, 0.29 g $NaHCO_3$, 100 ml de-ionized water, pH 9.6) and dried at 37°C. Each batch of plates was standardized with reference sera from two people who had received the full course of immunization with the UK vaccine.

It was thought that, as an enzyme, EF might be relatively unstable either to overnight drying out at 37°C or to pH 9.6 or both. In a series of preliminary tests, the drying out method was carried out using three different concentrations of EF- 24, 17 and 8.5 μg/ml- to detect differences attributable to the decay of EF during the drying, and using buffered saline (pH 7.5) as well as the high pH coating buffer in comparative trials. Coating was also done using a 17 μg/ml solution of EF in coating buffer held for 2 hours in covered plates at 30°C.

Marginally weaker signals were obtained on the plates coated with EF in buffered saline but, among the plates coated with EF in the high pH coating buffer, the different antigen levels and methods used gave indistinguishable readings. As well, therefore, as being consistent with the method used for PA and LF, it was more convenient to use the overnight drying out method (± 15 μg/ml of coating buffer, pH 9.6) for the tests reported here.

The peroxidise conjugated antibodies used for the ELISA tests in this study were commercial preparations (Dakopatts a/s, Copenhagen, Denmark) raised against all immunoglobulin types.

Guinea pig challenge

The challenge given to group I guinea pigs and the protection afforded were as published previously [7]. Challenges consisted of 500 or 1,000 spores of the "vaccine resistant" Ames,

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New Hampshire (NH) and penicillin resistant strains administered intramuscularly.

Group II guinea pigs were similarly challenged with 980-1,300 spores of the Ames strain administered intramuscularly.

Statistical analysis

Log_2 values of the antibody titers were compared as appropriate by Fisher's Exact test or Student's *t* test for the difference between two sample means.

Results

Human vaccinees

The anti-PA, anti-LF and anti-EF titers of the human vaccinees can be compared in Table 1. UK vaccinees developed antibodies to all three toxin components although anti-EF titers generally became detectable later and remained relatively lower than anti-PA and anti-LF titers. There was no evidence of the presence of anti-LF or anti-EF antibodies in the sera of the US vaccinees after their short courses.

Human case sera

Only two of the nine sera which had exhibited anti-PA titers (seven of which were also anti-LF positive) possessed measurable anti-EF titers (Table 2). A third showed slight evidence of the presence of antibodies of EF but below the level at which a titer could be assigned.

Titers in guinea pig sera

The findings with Group I guinea pigs are summarized in Table 3. In relating the titers in guinea pigs with those in humans in response to the human vaccines, it is to be noted that the guinea pig received human-size (0.5 ml) doses equivalent on a body weight basis to 100 to 200 times the dose administered to the human vaccinees.

The absence of detectable anti-EF antibody in the animals which received the US vaccine again indicates the virtual absence of this toxin component in that vaccine. One of the animals immunized with the UK vaccine developed the remarkably high titer of 1:16,400, but the remaining animals had low (1:32, 1:16) or negative titers.

The patterns of anti-PA, anti-LF and anti-EF responses to the human vaccines seen in the Group I guinea pigs were paralleled in the Group II animals (Table 4). Anti-PA titers were again higher overall in those that had received the US vaccine than in those given the UK vaccine but the difference was not statistically significant. Anti-LF activity, on the other hand, was again apparent at generally high levels in the groups that had received the UK vaccine but only at low levels or not at all in the US vaccine groups (4 of 15 with measurable

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titers 1:64 to 1:2,048 and 2 with evidence of antibody below that at which a titer could be assigned; the rest undetectable). Antibodies to EF were detected in 8 of the 16 guinea pigs that had received the UK vaccine with measurable titers in 6 but all those immunized with the US vaccine were negative for anti-EF antibody.

Table 1. Antibodies to PA, LF and EF in relation to current vaccination schedules in humans[a].

Time sera collected	Serum ID	Anti-PA titer	Anti-LF titer	Anti-EF titer
2 weeks after end	RT4	2,048	1,024	w[b]
of short course	PT4	1,024	1,024	Neg
(UK vaccinees)	DW4	2,048	512	Neg
	TH4	1,024	4,096	Neg
	TR4	1,024	2,048	Neg
	LM4	1,024	2,048	4
2 weeks after	RT6	8,192	8,192	2,048
6-month booster	PT6	16,400	8,192	Neg
(UK vaccinees)	DW6	4,096	512	Neg
	TH6	4,096	8,192	Neg
	TR6	4,096	2,048	256
	LM5	1,024	2,048	32
Months post annual booster (UK vaccinees)				
	RT6.8.86	4,096	8,192	512
0.5	PT4.11.86	4,096	2,048	128
	TR21.7.86	8,192	8,192	256
	JC	2,048	1,024	64
1.25	DY	8,192	1,024	128
3	BX	8,192	4,096	128
6	BQ	4,096	4,096	64
10	RT22.5.85	256	256	32
11	RT15.5.86	512	1,024	16
11	PT17.10.86	1,024	2,048	Neg
19	BN	8,192	1,024	128
58	AD	4,096	16,400	128
US vaccinees	US11	4,096	Neg	Neg
	US19	8,192	Neg	Neg
2 weeks after end	US23	128	Neg	Neg
of short course	US24	4,096	Neg	Neg
	US26	8,192	Neg	Neg
	US 28	32,800	Neg	Neg

[a] Titer expressed as the reciprocal of the dilution

[b] Weak- evidence of antibody but below level at which a titer could be assigned.

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Table 2. Antibodies to PA, LF and EF in sera from clinically diagnosed human cases of anthrax[a].

Serum ID	Anti-PA[b] titer	Anti-LF[b] titer	Anti-EF titer
N1/0	Neg	Neg	Neg
N3/7	Neg	Neg	Neg
N4/5	Neg	Neg	Neg
N9/0	512	Neg	Neg
N13/7	2,048	256	128
N21/0	2,048	256	Neg
N21/15	4,096	1,024	Neg
N21/42	1,024	128	Neg
N32/7	>16,400	8,192	2,048
N66/13	256	8,192	Neg
N82/0	1,024	Neg	Neg
N83/0	Neg	Neg	Neg
N118/V	128	1,024	w[c]

[a] Expressed as the reciprocal of the dilution

[b] A number of these were reported previously [7]

[c] Weak- evidence of antibody but below level at which a titer could be assigned

Among the guinea pigs (Group I, Table 3) receiving the standard single injection of live spore vaccine, at the time of challenge 6 weeks later, only one that had received the Sterne strain vaccine had a detectable anti-EF titer (1:256) while 5 of the 6 that had been immunized with the vaccine derived from Russian STI strain had measurable anti-EF levels. It is to be recalled though [7] that the STI vaccine was significantly more virulent than the Sterne strain vaccine and killed almost a third of the guinea pigs to which it was administered as a vaccine.

Although 6 weeks after challenge mean anti-EF titers had risen in the Sterne vaccine group, one of these animals still had undetectable anti-EF antibodies.

Antibody and protection

Protection test results and analysis on the Group I guinea pigs have already been reported [7]. Briefly, at the time of the first challenge, the Group I guinea pigs that had received the human vaccine were significantly less well protected ($P < 0.001$) than those that had received the live spore vaccine despite possessing generally higher levels of anti-PA and, in the group immunized with the UK vaccine, as high or higher anti-LF titers as well.

Examined as part of the present study, there were no significant differences (Table 3) in either the proportions of animals having detectable anti-EF antibody or the titers in the positive animals when the UK human vaccine group (protection rate 33%) was compared with the Russian STI live spore vaccine group (protection rate 72%). All (6/6) the US human

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Table 3. Antibodies to PA, LF and EF in vaccinated (Group I) guinea pigs[a]

Vaccine[b] administered	% survival in group[c]	Serum ID	Anti-PA titer	Anti-LF titer	Anti-EF titer
UK human	33	4B	32,800	32,800	32
		5B	8,192	65,600	16,400
		7B	16,400	16,400	Neg
		1B	32,800	65,600	Neg
		X124/A	4,096	8,192	16
		X11/13	16,400	4,096	Neg
US human	17	11B	16,400	Neg	Neg
		12B	65,600	256	Neg
		13B	65,600	64	Neg
		15B	32,800	4,096	Neg
		X124/J	16,400	Neg	Neg
		X11/9	32,800	256	Neg
Live spore (British)	65	1	256	64	Neg
		2	4,096	1,024	Neg
		3	1,024	256	Neg
		4	32,800	16,400	256
		5	1,024	256	Neg
		6	2,048	256	Neg
Live spore (Russian STI)	72	7	4,096	2,048	16
		8	8,192	16,400	16
		10	4,096	16,400	8,192
		11	8,192	8,192	256
		15	256	256	Neg
		16	8,192	16,400	16,400
Live spore (British) Re-challenged 6 weeks after 1st challenge	100	A	32,800	32,800	1,024
		B	16,400	32,800	8,192
		C	1.2×10^5	32,800	16,400
		D	32,800	32,800	256
		E	32,800	32,800	64
		F	32,800	16,400	Neg
Live spore (Russian STI) Re-challenged 6 weeks after 1st challenge	100	K	65,600	16,400	4,096
		I	32,800	8,192	65,600
		L	65,600	16,400	8,192
		J	32,800	16,400	2,048

[a] Titers expressed as the reciprocal of the dilution

[b] Human-type vaccines administered as 3 human-size doses (0.5 ml) at 3-week intervals. These animals were challenged after a further 2 weeks. Live spore vaccines were administered as a single dose and the animals challenged after 6 weeks.

[c] Survival against challenge with Ames, New Hampshire and Penicillin Resistant strains; all vaccines gave 100% protection against the Vollum strain.

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Table 4. Protection and antibodies to PA, LF and EF induced in Group II guinea pigs by the UK and US human vaccines[a]

Vaccine	Supplement[b]	Protection[c]	No. serologically tested	Anti-PA		Anti-LF		Anti-EF	
				Mean	Range	Mean	Range	Mean	Range
UK	Bc/48 *C. ovis* FCA	16/16	6	32,800	4,096-262,400	32,800	8,192-131,200	4	Neg-1,024
US	*C. ovis* FCA	11/11	4	264,200	131,200-524,800	Neg	—	Neg	—
UK	Bc/43	3/6	2	8,192	4,096 & 16,400	8,192	8,192 & 16,400	32	Neg & 1,024
US	Bc/48 Bc/43	4/12	4	16,400	8,192-16,400	16	2-128	Neg	—
UK	PBS Quil-A Gasil-23D	3/25	8	16,400	2,048-131,200	8,192	4,096-32,800	8	Neg-1,024
US	PBS Quil-A Gasil-23D	1/24	8	32,800	16,400-65,600	8	Neg-2,048	Neg	—
PBS	Bc/48 Bc/43 *C. ovis* FCA Quil-A Gasil-23D	0/53	12	Neg	—	Neg	—	Neg	—

[a] Titers expressed as the reciprocal of the dilution

[b] Bc/48 and Bc/43 = *Bacillus cereus* strains F4810/72 and F4433/73; FCA = Freund's complete adjuvant; PBS = phosphate buffered saline

[c] Challenged with 1,000 spores of the Ames "vaccine resistant" strain of *Bacillus anthracis*

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vaccine group (protection rate 17%) were negative for anti-EF antibody; in comparison, all but one (5/6) of the Sterne strain live spore vaccine group (protection rate 65%) were also negative for antibodies to EF. Although anti-EF titers were enhanced in the re-challenged live spore vaccine groups (protection rate 100%), the titers in the Sterne strain set ranged widely with one being negative and two fairly low (1:64; 1:256).

In the Group II guinea pigs (immunized with the human vaccines only), there were no significant differences between the anti-PA titers or the protection statuses when those given the UK vaccine (21/47, that is, 44.7%, survived) were compared with those immunized with the US vaccine (16/40, that is, 40%, survived). Anti-PA titers were significantly greater ($P < 0.0001$) in the fully protected (21/21 = 100%) animals given the US and UK vaccines supplemented with *Corynebacterium ovis* and Freund's complete adjuvant as compared with those given the vaccines supplemented with buffered saline or chemical adjuvants (protection rate 3/36 = 8.3%). However, when supplementation with *Bacillus cereus* was taken into account, the differences between the anti-PA titers of the supplemented and un-supplemented groups became statistically non-significant, while the differences in protection rates- 27/27 (100%) compared with 3/48 (6%)- remained highly significant ($P < 10^{-6}$).

In the case of both anti-LF and anti-EF, there was no obvious relationship between antibody and protection. The US vaccine supplemented with *Corynebacterium ovis* or Freund's complete adjuvant conferred 100% protection while failing to induce detectable anti-LF or anti-EF antibodies. Also, of the 16 fully protected guinea pigs that had been vaccinated with the microbially-supplemented UK vaccine, anti-EF antibodies were not detected in 2 of 6 sera randomly selected for testing.

Discussion

It was the prediction of the forerunning study on the relationship between anti-PA and anti-LF antibody and protection based on the anti-PA and anti-LF titers in the Group I guinea pigs [7] that effective protection against all strains of *Bacillus anthracis* will prove to be a function of more than just substantial antibody titers to PA and LF.

The extended work presented here demonstrates that protection is possible in the absence of detectable anti-LF and anti-EF antibodies. This is particularly apparent in the Group II guinea pigs immunized with the US vaccine supplemented with *Corynebacterium ovis* or Freund's complete adjuvant (Table 4). Anti-LF and anti-EF antibodies have, in fact, been detected by others [1] in guinea pigs in response to the US vaccine but only at the low mean ELISA titers of 1:3,311 and 1:49 respectively relative to a mean anti-PA titer of 1:64,508.

PA appears to be essential to protection; protection has not been achieved without it. Furthermore, direct evidence has now been supplied by the results of a study [2] in which substantial protection was afforded to guinea pigs immunized with strains of *Bacillus subtilis* cloned with the PA gene, able to produce PA but not LF or EF. Nevertheless, the relationship between PA and protection is not straightforward; in the final analysis, there was no significant difference between the anti-PA antibody titers of those animals fully protected by microbially-supplemented human vaccines and those that were poorly or not protected by these vaccines supplemented with buffered saline or chemical adjuvants. It remains apparent that high anti-PA titers can still be associated with poor protection.

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Our interim conclusion is, therefore, that PA probably is the essential anthrax-derived antigen for the protective action of the current vaccines but correct presentation to the host's immune system is critical to induction of full protective immunity. The human vaccines are essentially crude culture filtrates and the possibility that some other undescribed metabolites of *B. anthracis* present in them may play a role in protection has not been conclusively ruled out yet. If such a metabolite exists, it is not one shared by the closely related *Bacillus cereus* which, in the absence of PA failed to protect against challenge with the Ames strain (Table 4).

PA purified to single SDS-PAGE band purity is now available [5] and conclusive evidence of the protective role of PA awaits the outcome of protection tests on microbially supplemented pure PA.

The stability of the enzymic EF for ELISA purposes was the subject of some initial concern and it was necessary to rule this out as a reason for low or negative titers in early tests. However, identical titers in a selected group of the sera in a series of tests using different coating schedules and the development of titers as high as found with PA and LF in repeatedly exposed guinea pigs (Table 3) resulted in confidence that the ELISA system was performing accurately in detection of anti-EF antibody. EF appears, therefore, to possess a stable antigenic portion suitable for ELISA purposes.

The inhibition ELISA was also used to test for cross-reactivity between the PA, LF and EF preparations used. A one-way cross-reaction was found in which anti-EF antibodies were inhibited by LF but not vice versa. No other cross-reactions were noted. Thus neither anti-PA nor anti-LF antibodies could have accounted for the anti-EF titers observed. Anti-EF antibodies, where present, may have contributed to the anti-LF titers but were clearly of negligible consequence.

The enhanced protection conferred by the microbial supplements probably results from their stimulation of the cellular immune system concurrent with the humoral response to PA. This may account for the greater efficacy of the live spore animal vaccine as compared with the human vaccines (Table 1) [1, 7]; the bacteria of the live spore vaccine presumably stimulate the host's cellular immunity at the same time as producing PA during their brief period of infection following injection. The cloned *B. subtilis* IS53 carrying the recombinant genes for production of PA [2] offers similar potential for enhanced protective efficacy through combined production of PA and microbial stimulation of cell-mediated immunity.

In summary, evidence is supplied in this paper that protective immunity to anthrax is a function of more than just substantial antibody titers to the three anthrax toxin components and measurable anti-PA, anti-LF and/or anti-EF titers are not reliable indicators of certain protected status.

The results presented further show that, in a future, second generation, subunit vaccine, PA will be an essential ingredient. However, PA is a log phase metabolite; antibodies to PA induced by a vaccine are directed against the action of the toxin and not at the multiplying *B. anthracis* in an infection. Ideally, a vaccine should contain a protective antigen which elicits antibody targeted at the spore or germinating cell- i.e., acting early in the infection. The search for such an antigen is in progress.

The non-specific stimulation of protection by microbial supplements demonstrated here may, in fact, occur as a result of a generally enhanced alertness of the host's immune system to

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the earlier stages of infection, but this enhancement may be one of limited duration only. The duration of protection afforded by various formulations is also under examination now.

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